

Effects of Low-Temperature Acclimation and Oxygen Stress on Tocopherol Production in *Euglena gracilis* Z

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The effects of low-temperature acclimation and oxygen stress on tocopherol production were examined in the unicellular phytoflagellate *Euglena gracilis* Z. Cells were cultured photoheterotrophically at $27.5 \pm 1^\circ\text{C}$ with 5% carbon dioxide-95% air and $740 \text{ microeinsteins m}^{-2} \text{ s}^{-1}$ (photosynthetically active radiation) and served as controls. Low-temperature acclimation ($12.5 \pm 1^\circ\text{C}$) and high-oxygen stress (5% carbon dioxide-95% oxygen) were individually examined in the mass culturing of the algae. Chromatographic analyses demonstrated a six- to sevenfold enhancement of α -tocopherol production in temperature-stressed cells, along with a concomitant decline in the levels of α -tocotrienol and the absence of other tocopherol homologs. Oxygen-stressed cultures demonstrated the presence of high levels of α -tocopherylquinone; α -tocopherol and its homologs and precursors were absent or declined markedly. These findings are discussed in terms of the feasibility of microbial production of natural tocopherols. In addition, these results lend themselves to speculation regarding the biological role(s) of tocopherols as antioxidants and free radical scavengers in reducing photo-induced oxidative damage or lipid peroxidation toxicities or both in photosynthetically active *E. gracilis* Z.

The nutritional and biochemical roles of vitamin E (α -tocopherol and its homologs) as an antioxidant and free radical scavenger (17, 24) and a membrane lipid stabilizer (6, 15) have become increasingly recognized. In an attempt to determine novel economical sources of natural tocopherols and related quinones, the mass fermentation of these compounds from microbial sources has become a biotechnological problem worthy of investigation.

In the past a variety of microbial flora have been exploited commercially for the production of water-soluble vitamins and carotenoids (1, 3, 11), yet comparatively little attention has been directed toward microbial tocopherol production and its feasibility. Tocopherol production in microorganisms has recently been reviewed and examined in a number of procaryotic and eucaryotic organisms (9; B. A. Ruggeri, M.S. thesis, University of Delaware, Newark, Del., 1984). These investigations found that most procaryotes and yeasts produce little if any α -tocopherol, its homologs, or commercially valuable quinone moieties. Among the most promising tocopherol-producing species investigated in this and other laboratories (8, 9, 14, 26; K. K. Shiseido, Japanese patent 128769, Sept. 1980) is the unicellular phytoflagellate *Euglena gracilis* Z. This genus of the Euglenophyta possesses a number of stable taxonomic forms inhabiting diverse, often harsh environments (12). The nutritional and biochemical versatility of this genus, particularly its lipid biosynthetic capabilities, have been previously demonstrated (4, 10, 13, 20). The type species of the genus *E. gracilis* Z is capable of growing as a strict autotroph, a photoheterotroph, or a strict heterotroph lacking differentiated chloroplasts; the lipid profile of the species reflects its nutritional mode (4, 13, 20).

The levels and types of tocopherols and terpenoid quinones demonstrated to date in members of this genus are summarized in Table 1.

The present study was directed at investigating the effects of low-temperature stress and altered oxygen tension on the production of tocopherols in photoheterotrophic cultures of *E. gracilis* Z. In an attempt to enhance production of the vitamin in mass culture, these manipulations were directed at increasing the probability of oxidative or photoinduced free radical damage or both to the cells, with photoinduced free radical damage resulting from low-temperature or oxidative stress (18, 21). Because the net effect of these cellular perturbations would be a greater physiological need for a membrane-localized antioxidant-free radical scavenger-lipid-stabilizing agent, we attempted to stimulate the tocopherol biosynthetic capabilities of the alga in responding and adapting to these environmental stresses.

MATERIALS AND METHODS

Culture conditions. *E. gracilis* Z was obtained from the culture collection of the Department of Botany, University of Texas (Austin). Cultures were plated on nutrient agar plates to screen for bacterial or fungal contaminants. Stock cultures (10 ml) were maintained in *Euglena* broth (Difco Laboratories, Detroit, Mich.) at 23 to 25°C on a light bench illuminated by two 40 W Cool-White fluorescent lamps placed 35 cm from the cultures. Stocks were transferred every 4 months to fresh medium.

Starter cultures (100 ml) of *E. gracilis* Z in *Euglena* broth (Difco) were incubated at $27.5 \pm 1^\circ\text{C}$ under atmospheric conditions in a Psychrotherm controlled environment unit (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). Stationary cultures were illuminated with $740 \text{ microeinsteins m}^{-2} \text{ s}^{-1}$ photosynthetically active radiation (PAR) provided by eight Cool-White fluorescent lamps positioned 35 cm above the cultures.

Algal growth was monitored turbidimetrically at 540 nm and harvested at an optical density of 1.15 to 1.30. Inoculation of growth medium was at a 2% level in six 1-liter volumes of *Euglena* broth sterilized in 2.2-liter low-neck

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TABLE 1. Summary of published data on tocopherol and terpenoid quinone levels present in various strains of *E. gracilis*^a

Strain (variety) examined	α -Tocopherol level (unless specified)	Plastoquinone level	α -Tocopherylquinone level	Reference or source
Z ^b	155	ND ^c	ND	8
Z	701 \pm 72 SEM ^d	1,983 \pm 264 SEM	421 \pm 72 SEM	27
Z	α -Tocopherol, 3,200 μ g/liter γ -Tocopherol, 1,000 μ g/liter δ -Tocopherol, 800 μ g/liter	ND	ND	e
Z	797	ND	ND	14
Z	ND	ND	3.5 2.5 ^f	9
1224	340	1,344	212	26
var. <i>bacillaris</i>	378 \pm 58 SEM	677 \pm 15 SEM	353 \pm 93 SEM	26

^a Values described (micrograms per gram of dry weight of cells unless specified) are for cells cultured photoheterotrophically unless noted.

^b Etiolated cultures.

^c ND, Not detected or specified.

^d Standard error of the mean of three observations.

^e Shiseido, Japanese patent 128769, 1980.

^f Cells grown under bleached conditions.

Fernback flasks (Corning Glass Works, Corning, N.Y.). Cultures were incubated at $27.5 \pm 1^\circ\text{C}$ under filtered 5% carbon dioxide-95% air (set at a flow rate of 900 cc/min) in the Psychrotherm unit. Cells were agitated at 60 rpm and illuminated as above with 740 microeinsteins $\text{m}^{-2} \text{s}^{-1}$ (PAR) as determined by a Li-Cor Li-185A Quantum radiometer. Growth was monitored turbidimetrically at 540 nm.

Actively growing cultures (1- to 2-liter volumes) were harvested at 27.5, 55, 57.5, 100, and 125 h and served as controls. Duplicate experimental trials were conducted.

Low-temperature studies. Cultures used in these studies were grown under identical incubation conditions as those described above and in duplicate trials. After 55 h of incubation a sample of cells was harvested, and the temperature within the incubation chamber was lowered from $27.5 \pm 1^\circ\text{C}$ (control) to $12.5 \pm 1^\circ\text{C}$. This was accomplished by decreasing the temperature 2 to 3°C at 15-min intervals over 2.5 h. At this time (57.5 h) additional cells were harvested. Finally, low-temperature-acclimated cells were harvested at 100 and 125 h. All incubation parameters except temperature were identical to those of controls.

Oxygen-stress studies. Cultures of *E. gracilis* Z were prepared and incubated as described for controls. At 55 h the atmosphere was modified to a filtered mixture of 5% carbon dioxide-95% oxygen; flow rate, temperature, agitation, and illumination were maintained as in controls. Cultures were harvested at 55 h (i.e., before atmosphere modification) and at 57.5, 100, and 125 h. Oxygen-stress trials were done in duplicate.

Harvesting and lipid extraction procedures. Algal cultures were harvested by centrifugation at $330 \times g$ for 15 min at 0 to 5°C . Supernatants were discarded, and cell pellets were washed twice with distilled water, suspended, and transferred to preweighed freeze-drying vessels. After freezing at -20°C , samples were lyophilized with a freeze-drier (Labconco Corp., Kansas City, Mo.). After 36 h of freeze-drying, the vacuum was released under nitrogen and sample dry weights were obtained.

Solvents and reagents used for extraction purposes were either spectral grade or high-pressure liquid chromatography grade. Samples were extracted by a slight modification of the method of Bligh and Dyer (2) with a small volume of benzene in addition to the chloroform-methanol-water (2:1:0.8, vol/vol/vol) system. All extractions were conducted in darkened flasks flushed with nitrogen with mild vortexing at room temperature. Chloroform extracts were filtered (0.45- μm

membrane filter; Millipore Corp., Bedford, Mass.) and reevaporated to dryness under vacuum at $35 \pm 2^\circ\text{C}$ with a Büchi rotary evaporator. Extracts were suspended in several volumes of chloroform, divided equally into preweighed conical flasks, and evaporated as above. Extracts were used for gravimetric determination of total lipid and tocopherol analyses, respectively; the latter samples were suspended in several milliliters of methanol or acetone for chromatographic analyses. Sample tubes were flushed with nitrogen and stored at -20°C . Analyses were conducted within 5 days of storage.

Chromatographic analyses. The standard compounds used in this investigation were *dl*- α -tocopherol, *dl*- γ -tocopherol, *dl*- δ -tocopherol, *dl*- α -tocotrienol, *dl*-tocol, and *dl*- α -tocopherylquinone. Standards were a gift from Hoffmann-La Roche Inc. (Nutley, N.J.).

Thin-layer chromatography. Silica gel GF plates (20 cm \times 20 cm \times 250 μm silica) were obtained from Analtech (Newark, Del.). Plates were prewashed in chloroform-methanol (1:1, vol/vol) and after drying were activated at 100°C for 10 min. Samples and standards were applied under diffuse light in volumes ranging from 0.5 to 2.0 μl . Plates were developed unidirectionally in a preequilibrated tank in a mobile phase of hexane-isopropyl ether (85:15, vol/vol) as previously described (22). Plates were visualized by saturation with 10% cupric sulfate-8% phosphoric acid followed by charring at 200°C for 10 min. The resolved bands were quantitated densitometrically (absorbance mode) with a Shimadzu high-speed thin-layer chromatography scanner (model CS-920) set at 350 nm with a deuterium lamp.

High-performance liquid chromatography. For purposes of verification, random samples were analyzed by high-performance liquid chromatography with a reversed-phase Varian MCH 10 C18 Micropak column and an isocratic mobile phase of methanol-water (95:5, vol/vol) as described previously (22). Both chromatographic systems demonstrated a comparable 92% recovery of α -tocopherol (22).

Data were analyzed by the two-tailed Dunnett test as described by Zar (28).

RESULTS AND DISCUSSION

The concentrations of tocopherols and related compounds found in *E. gracilis* Z under control and low-temperature conditions and the time-dependency for both concentration and type of homolog are shown in Tables 2 and 3. It appears that α -tocopherol levels in control cultures (Table 2) peak at

TABLE 2. Tocopherol and tocotrienol production by *E. gracilis* Z control cultures

Harvest time (h)	Compound assayed	Yield ^a	
		Cell dry wt (μg/g)	Total lipid (μg/mg)
27.5	α-Tocotrienol	2,029 ± 250	11.1 ± 0.30
	α-Tocopherol	ND ^b	
55	α-Tocotrienol	2,484 ± 360	6.3 ± 0.30
	α-Tocopherol	4,664 ± 180	24.6 ± 4.5
57.5	α-Tocotrienol	2,990 ± 250	9.9 ± 0.15
	α-Tocopherol	4,365 ± 200	12.5 ± 0.15
100	α-Tocotrienol	482 ± 20	8.6 ± 0.35
	α-Tocopherol	640 ± 35	10.6 ± 0.23
125	α-Tocotrienol	337 ± 35	5.0 ± 0.52
	α-Tocopherol	1,070 ± 55	15.9 ± 0.80

^a Yield was corrected for 92% recovery of α-tocopherol by using the analytical procedures described in the text. Values are mean plus or minus standard deviation.

^b ND, Not detected.

approximately 55 to 57.5 h and then decline with culture age. Whether this decline is a result of increased catabolism, reduced biosynthesis, or both remains to be elucidated. Further experimental trials with sampling at shorter intervals are needed to ascertain the optimal period for α-tocopherol biosynthesis.

The concentrations reported here for 100- and 125-h control cultures fall within the range of those reported previously for *E. gracilis* Z (14, 26; Shiseido, Japanese patent 128769, 1980; Table 1), whereas values obtained for cells in early to midexponential growth are much higher. Litton and Gilbert (14) failed to provide sufficient information on illumination conditions or harvesting times in their studies. Similarly, the results reported by Threlfall and Goodwin (26) are based on 7-day cultures grown photoheterotrophically, but under different conditions of illumination, aeration, and agitation, all of which were not clearly specified. Consequently, precise comparisons with the findings reported here are not possible.

The effect of low-temperature acclimation of *E. gracilis* Z is shown (Fig. 1). It can be seen that throughout the acclimation period, cell division ceased for approximately 50

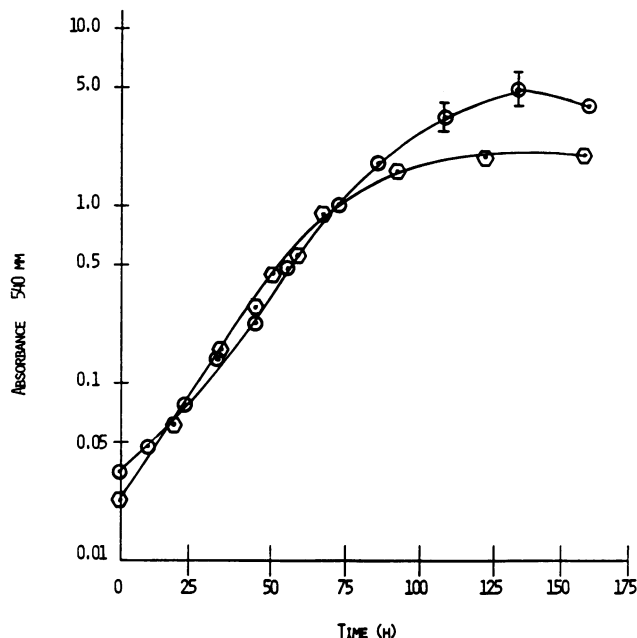


FIG. 1. Growth curves of *E. gracilis* Z under control and low-temperature-acclimated conditions for control (○) (27.5 ± 1°C) and low-temperature-acclimated cultures (Δ) (12.5 ± 1°C). Incubation conditions: atmosphere, 5% carbon dioxide-95% air; agitation, 60 rpm; illumination, 740 microeinsteins m⁻² s⁻¹ (PAR); gas flow rate, 900 cc/min; medium, 1-liter volumes of Euglena broth in 2,200-ml flasks; inoculum, 2% from 3-day-old starter cultures (optical density, 1.15 to 1.30 at 540 nm). Generation time of control, 16.4 h; μ, 0.042 h⁻¹. I denotes range in absorbance readings.

h; growth then resumed but at a reduced rate. This pattern is similar to that reported for the halotolerant alga *Dunaliella salina* (16) grown at comparable temperatures, although the latency period for the latter organism was approximately twice as long.

The effects of low temperature on tocopherol production were marked (Table 3). It would appear that despite the slight decline in α-tocopherol production in the first few hours of low-temperature exposure, during the acclimation period (ca. 50 h) α-tocopherol production was enhanced significantly. Concentrations approximately 11-fold greater than 100-h controls (Table 2), and concentrations 6-fold greater than those of 125-h controls were observed. Although no reports of this nature have appeared, Fork (5) observed a marked increase in carotenoid production in *Synechococcus lividus* under comparable conditions of low-temperature acclimation.

An important observation in these results was the pronounced inverse relationship demonstrated between elevated α-tocopherol levels in low-temperature cultures and the absence of any detectable level of α-tocotrienol in these cultures (Table 3). Although the authors are not aware of any prior reports of this moiety in *E. gracilis*, these findings are in agreement with several of the biosynthetic scenarios for tocopherol production (7, 25) in other species studied.

The effect of oxygen stress on *E. gracilis* Z is illustrated (Fig. 2). It can be seen that the generation time of the cultures was not affected by atmospheric modification, the μ value being almost identical with that of control cultures throughout mid- to late-exponential growth.

The effects of oxygen stress on tocopherol production in

TABLE 3. Tocopherol and tocotrienol production by *E. gracilis* Z low-temperature-acclimated cultures

Harvest time (h)	Compound assayed	Yield ^a	
		Cell dry wt (μg/g)	Total lipid (μg/mg)
55	α-Tocotrienol	2,450 ± 300	6.2 ± 0.18
	α-Tocopherol	4,620 ± 140	24.4 ± 4.2
57.5	α-Tocotrienol	2,618 ± 350 ^b	13.0 ± 0.20
	α-Tocopherol	3,971 ± 250	19.8 ± 0.10
100	α-Tocotrienol	ND ^c	
	α-Tocopherol	7,214 ± 100 ^d	64.8 ± 2.0
125	α-Tocotrienol	ND	
	α-Tocopherol	6,729 ± 500 ^e	55.5 ± 2.7

^a Yield was corrected for 92% recovery of α-tocopherol by using the analytical procedures described in the text. Values are mean plus or minus standard deviation.

^b Significant at *P* < 0.05 compared with control values (Table 2).

^c ND, Not detected.

^d Significant at *P* < 0.001 compared with control values (Table 2).

^e Significant at *P* < 0.025 compared with control values (Table 2).

E. gracilis Z are shown (Table 4). The 50% decline in α -tocopherol production in a 2-h period is somewhat puzzling, yet it was observed in both trials. Oxygen-stressed cultures demonstrated no detectable levels of tocopherol homologs or their precursors in contrast to the findings reported here and elsewhere (8, 14, 26; Shiseido, Japanese patent 128769, 1980) for nonstressed cultures. The presence of only α -tocopherylquinone, the natural oxidation product of α -tocopherol, was observed in high concentrations. Hughes and Tove (9) and Threlfall and Goodwin (26) have reported the presence of α -tocopherylquinone in *E. gracilis*, whereas others (8, 14; Ruggeri, M.S. thesis; Shiseido, Japanese patent 128769, 1980) failed to observe this moiety in cells grown under nonstressed conditions.

The mechanisms by which low temperature enhance α -tocopherol production or the exact role(s) of the compound in low-temperature adaptation are uncertain. A major effect of low temperature on photosynthesis, however, is to excite the photosynthetic apparatus, particularly photosystem II, to such an extent that protective mechanisms can no longer prevent photo-induced, free radical-mediated damage from occurring (18). Partial destruction of reaction centers and disorganization of the chloroplast ultrastructure have likewise been observed under such conditions (18). The possibility that enhanced synthesis of α -tocopherol in the chloroplast (23) may play a role in protecting thylakoid components from oxidative damage must be considered. Alternatively, the proposed roles of α -tocopherol in photosynthetic electron transport (19, 27) and photophosphoryla-

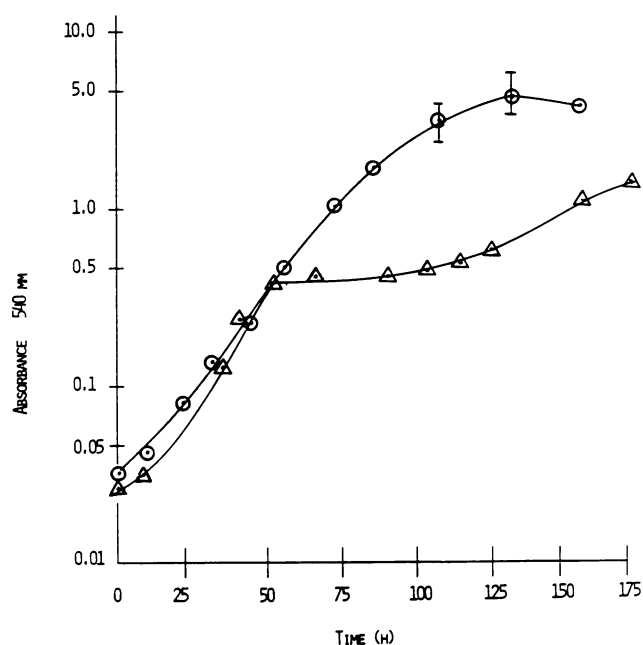


FIG. 2. Growth curves of *E. gracilis* Z under control and oxygen-stressed conditions for control (○) (5% carbon dioxide-95% air) and oxygen-stressed cultures (△) (5% carbon dioxide-95% oxygen). Incubation conditions: temperature, $27.5 \pm 1^\circ\text{C}$; agitation, 60 rpm; illumination, $740 \mu\text{E m}^{-2}\text{s}^{-1}$ (PAR); gas flow rate, 900 ml/min; medium, 1-liter volumes of Euglena broth in 2,200-ml flasks; inoculum, 2% from 3-day-old starter cultures (optical density, 1.10 to 1.20 at 540 nm). Generation time of control, 16.4 h; μ , 0.042 h^{-1} ; generation time of oxygen-stressed culture, 17.3 h; μ , 0.040 h^{-1} . \pm denotes range in absorbance readings.

TABLE 4. Tocopherol and tocotrienol production by *E. gracilis* Z oxygen-stressed cultures

Harvest time (h)	Compound assayed	Yield ^a	
		Cell dry wt ($\mu\text{g/g}$)	Total lipid ($\mu\text{g/mg}$)
55	α -Tocotrienol	2.446 ± 320	6.3 ± 0.12
	α -Tocopherol	4.650 ± 310	24.7 ± 5.1
57.5	α -Tocopherylquinone	2.692 ± 400	12.3 ± 0.18
	α -Tocopherol	ND ^b	
100	α -Tocotrienol	ND	
	α -Tocopherylquinone	5.800 ± 850	54.5 ± 4.35
	α -Tocopherol	ND	
125	α -Tocotrienol	ND	
	α -Tocopherylquinone	5.750 ± 715	55.6 ± 0.75
	α -Tocopherol	ND	
	α -Tocotrienol	ND	

^a Yield was corrected for 92% recovery of α -tocopherol by using the analytical procedures described in the text. Values are mean plus or minus standard deviation.

^b ND, Not detected.

tion (27) may be operative in the maintenance of photosynthetic capacity under the stresses imposed by low temperature. These hypotheses await clarification.

Similarly, the findings reported here of the presence of high levels of α -tocopherylquinone in oxygen-stressed *E. gracilis* Z lend support to the possibility that α -tocopherol may function in an antioxidant capacity in protecting against elevated oxygen levels or the peroxidation products resulting secondarily from this stress or both. The adaptability of *E. gracilis* Z to a range of oxygen tensions has been previously demonstrated (12). Future studies are warranted in examining the effects of varying oxygen levels on tocopherol production and metabolism in this species.

The finding that relatively simple alterations in certain parameters in the culture conditions of *E. gracilis* Z leads to enhanced production of α -tocopherol or its metabolites is significant from both a physiological and biotechnological perspective. Although the exact mechanism of this effect remains to be elucidated, significant increases in the production of α -tocopherol or its less active homologs help improve the cost-effectiveness of the fermentation of natural tocopherols relative to the energy input involved. Despite these experimental findings and the excellent yields of biomass obtained under the culture conditions used, the feasibility of large-scale commercial tocopherol production by *E. gracilis* Z is at present limited. The cost-effectiveness of this operation must be evaluated in terms of the cost of defined growth media, the energy input for optimal illumination and aeration, the fermentation apparatus, and the harvesting facilities. In addition, the labor intensiveness of the extraction and purification of tocopherols must be considered. Advances in biotechnological applications, including cell immobilization-permeabilization technologies and supercritical extraction procedures, may reduce the labor intensiveness of the extraction and purification processes, but these advances add additional burdens in terms of the equipment and energy input involved.

In conclusion, the findings reported here for *E. gracilis* Z shed new information on the possible biological roles of tocopherols in microorganisms analogous and in addition to those in mammalian species. Second, these findings demonstrate the potential for using and manipulating this species for future biotechnological application in the production of natural tocopherols.

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